MICROBIOLOGICAL REVIEWS, Dec. 1981, p. 542-555
0146-0749/81/120542-14$02.00/0

Vol. 45, No. 4

Succinate Dehydrogenase—a Comparative Review

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INTRODUCTION AND SCOPE

Membrane-bound succinate dehydrogenase [SDH; E.C.1.3.99.1 succinate:(acceptor) oxidoreductase] is present in all aerobic cells. Ever since its discovery in 1909 (93), SDH has been studied intensively. The enzyme has several particularly interesting properties: (i) SDH is a membrane-bound dehydrogenase linked to the respiratory chain and a member of the Krebs cycle; (ii) its activity is modulated by several activators and inhibitors; and (iii) SDH is a complex enzyme containing nonheme iron, acid-labile sulfur, and covalently bound flavin adenine dinucleotide (FAD).

Most of the published work concerns mammalian SDH. There is considerable knowledge about the composition, enzymology, and membrane binding of the enzyme, but relatively little is known about its genetics and biosynthesis. Mitochondrial SDH has been extensively reviewed (3, 37, 70, 87, 88), and only the structure and some new findings on the membrane binding of SDH will be discussed in this article. Compared with mitochondrial SDH, little is known about the corresponding procaryotic enzyme. However, we feel that sufficient knowledge on the comparative biology, genetics, membrane binding, and biosynthesis of microbial SDH has now accumulated that a short review would be of value. SDH catalyzes the oxidation of succinate to fumarate and transfers the resultant reducing equivalents directly to the respiratory chain. The enzyme is a member of both the Krebs cycle and the respiratory chain. In bacteria, the electron transport chains are located in the cytoplasmic membrane or in modifications thereof, like the chromatophore membrane of photosynthetic bacteria (55).

Fumarate reductase is often found in anaerobic or facultative organisms, where it reduces fumarate to succinate in the reverse of the SDH reaction. Fumarate reductase can be membrane bound and participate in anaerobic respiration with fumarate as the terminal electron acceptor, or it can be a soluble enzyme localized in the cytoplasm (85). SDH and fumarate reductase catalyze the same reactions, but their equilibriums are shifted toward succinate oxidation and fumarate reduction, respectively. In organisms like *Escherichia coli* containing both SDH and a membrane-bound fumarate reductase, the former enzyme is repressed during anaerobic growth, and the latter is repressed during aerobic growth (45, 89). SDH is also repressed to various extents during aerobic growth on glucose in several bacteria (68, 81).

Certain organisms contain enzymes with catalytic properties somewhere between SDH and fumarate reductase (85). This review will be restricted to SDH, a membrane-bound enzyme whose primary function is oxidation of succinate to fumarate. Membrane-bound fumarate reductase was reviewed recently by Kröger (57).

DETERMINATION OF SUCCINATE DEHYDROGENASE (SDH) ENZYME ACTIVITY

SDH activity is conveniently assayed by the succinate-dependent reduction of artificial electron acceptors, usually dyes which change color when reduced (3). In the most widely used assay, reduction of 2,6-dichlorophenol-indophenol (DCIP), with 5-*N*-methyl phenazonium sulfate (PMS) as intermediate electron carrier, is measured. However, SDH is inhibited at high PMS concentrations. To estimate maximal activity it is thus important to measure activity with increasing PMS concentrations and extrapolate to

infinite concentration (86). PMS and also Würsters blue (a semiquindimine radical of *N,N, N',N'*-tetramethyl phenylendiamine) (2, 3, 98) can accept electrons not only directly from SDH, but also from other components of the respiratory chain.

DCIP and dyes like methylene blue accept electrons from respiratory chain components downstream from SDH and not at the level of the enzyme. If PMS is excluded when membrane-bound SDH activity is measured with DCIP, the results become quantitatively different, e.g., the velocity of transport of electrons to the redox components of the respiratory chain that are electron donors to DCIP and their concentration in the membrane will affect the velocity by which DCIP is reduced by succinate. Ferricyanide has also been used to measure SDH activity. The soluble purified reconstitutively active enzyme has two types of ferricyanide reducing activities, a "low-K_m site" and a "high-K_m site" (96). Only the high-K_m site is expressed in the membrane-bound enzyme (see below, Reconstitution of Membrane-Bound SDH).

Due to the vectorial structure of the membrane, the diffusion barrier and the asymmetrical distribution of membrane proteins, the activity measurements of membrane-bound enzymes like SDH are more complicated than those of soluble enzymes. Membrane preparations which contain vesicles with unknown orientation of SDH are often used. Sealed membrane vesicles have permeability barriers to substrate and also electron acceptors. Most of the activity determinations made on bacterial SDH are confounded by permeability barriers. Inefficient electron acceptors or efficient acceptors at a single concentration have often been used. Much of the published results on the enzymology of bacterial SDHs has to be interpreted with care (34, 50, 53, 72, 77).

The succinoxidase of mitochondrial (33) and of bacterial (79) respiratory chains can be fragmented into segments or complexes that each show electron transfer activity and that can often be reconstructed into a functional succinoxidase. Succinate-ubiquinone (Q) reductase (often called complex II) contains SDH and is the most proximal segment of the succinoxidase. Succinate-Q reductase can be measured by reduction of DCIP with a quinone as intermediate electron acceptor.

ORIENTATION OF SDH IN MEMBRANES

SDH is the only membrane-bound enzyme of the Krebs cycle in both bacteria and mitochondria. Membranes are not freely permeable to dicarboxylic acids. As the substrate, succinate, is produced and the product, fumarate, is metabolized in the cytoplasm it is likely that the active site of SDH is located on the cytoplasmic side of the membrane. The orientation of SDH in membranes from various species has been determined by different methods. The results are compiled in Table 1.

The most convincing results on the orientation of SDH are those in which sealed membranes of both orientations, i.e., "right side out" and "inside out" have been used. Right-side-out bacterial membranes are easily obtained in the form of protoplasts. Chromatophore membrane

TABLE 1. *Orientation of SDH in bacterial and mitochondrial membranes*

Organism or organelle	Orientation	Method used to determine orientation	Reference
Bacillus subtilis	Outside and inside	Electron acceptors and trypsin treatment	56
	Inside	Antibody adsorption	Hederstedt et al., unpublished data
Micrococcus lysodeikticus	Inside	Antibody adsorption	74
Rhodospirillum rubrum	Inside	Trypsin and α-chymotrypsin treatment, enzymatic iodination	66
Rhodopseudomonas sphaeroides	Inside	Electron acceptors and trypsin treatment	91
	Inside	Antibody adsorption	26
	Inside	Reconstitution of succinoxidase from soluble SDH and membrane vesicles	47
Beef heart mitochondria	Matrix side	DABS labeling and antibody adsorption	63
	Matrix side	Reconstitution of succinoxidase from soluble SDH and alkali-treated ETP, electron acceptors, electron spin resonance, and availability to succinate	Review in 24

vesicles and mitochondrial electron transport particles (ETP) are examples of mainly inside-out membranes. When the orientation of the membrane is known, the sidedness of SDH can be investigated by several techniques.

One technique involves the use of different electron acceptors that can accept electrons at the level of the enzyme (and preferably at the same site) but have different membrane permeabilities. Enzyme activity is then measured with the different acceptors on a membrane preparation with a known orientation. Another approach to studying the sidedness of SDH is to use a membrane-impermeable electron acceptor such as ferricyanide (54). These kinds of experiments thus indicate on which side of the membrane the electron donor site(s) is located. Proteolytic enzymes, impermeable to membranes, that degrade SDH have also been used to establish sidedness. Fragmentation of SDH is detected by loss of enzymatic activity or loss of specific polypeptides or both. Adsorption of antimembrane antibody by various membrane preparations and subsequent qualitative and quantitative analysis of the unadsorbed antibodies by crossed immunoelectrophoresis have been successfully used to elucidate the orientation of SDH and other membrane-bound components (26, 73, 74). Immunoprecipitates containing SDH can be identified by zymogram staining. Unfortunately, the specificity of the antibodies that give rise to SDH-staining immunoprecipitates in crossed immunoelectrophoresis and the composition of the respective antigen are unknown in most cases. It is possible that the antibody reacts with membrane components that are attached to SDH in the detergent-solubilized enzyme. It is then the orientation of the attached components in the membrane that is determined. SDH-staining immunoprecipitates may show heterogeneity (26, 74), indicating inefficient solubilization or proteolytic modification.

SDH has, to our knowledge, been located exclusively on the inside (cytoplasmic or matrix) of the membrane in bacteria and mitochondria in all studies except one (56) (Table 1).

In these experiments a single type of membrane preparation from *Bacillus subtilis* was studied. The orientation of SDH was determined by using two electron acceptors, PMS and 5-*N*-methyl phenazonium-3-sulfonate with different membrane permeabilities. Both were assumed to accept electrons directly from SDH. However, it has later been shown that at least PMS can accept electrons from respiratory strain components downstream from SDH (7). The results of antibody adsorption experiments with *B. subtilis* intact protoplasts, lysed protoplasts, and Triton X-100-solubilized membranes indicate that SDH is located exclusively on the inside of the membrane (Hederstedt et al., unpublished data).

STRUCTURE

The simplest bacterial enzyme preparation with SDH activity was purified from *Rhodospirillum rubrum* chromatophore membranes by Hatefi and co-workers (22). SDH was released from the membranes with the chaotropic ion perchlorate in the presence of succinate and dithiotreitol as protective agents. The enzyme was fractionated and concentrated by $AmSO_4$ precipitation. The purified enzyme is water soluble and has a molecular weight on gel filtration of about 100,000. It contains covalently bound FAD, nonheme iron, and acid-labile sulfur (Table 2). Its composition is very similar to the enzyme isolated from beef heart mitochondria (21). Both contain equimolar amounts of two unequal subunits noncovalently bound to each other. The larger subunit, M_r 60K and 70K,

TABLE 2. *Composition of purified SDH*

Organism or organelle	Component	Mol wt (K)	Covalently bound FAD (mol/mol of protein)	Nonheme Fe (mol/mol of protein)	S^X (mol/mol of protein)	Ratio (mol/mol)	Polarity index (%)[a]
R. rubrum	SDH	100[b]	1	8	8		
	Fp	60[c]	1	Present	Present	1	43
	Ip	25[c]	0	Present	Present	1	42
Beef heart mitochondria[d]	SDH	110[b]	1	8	8		
	Fp	70[c]	1	4	4	1	44
	Ip	27[c]	0	4	4	1	48

[a] Calculated as the sum of the mole fractions of polar amino acids in the polypeptide as described by Capaldi and Vanderkooi (10).

[b] Molecular weight determined by gel filtration.

[c] Molecular weight determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

[d] Beef heart mitochondria data from references 16, 21, and 80.

respectively, contains covalently bound FAD. This subunit is called Fp (flavoprotein). The smaller subunit, M_r 25K and 27K, respectively, is called Ip (iron protein). Upon repeated freeze-thawing of SDH in the presence of sodium trichloroacetate the subunits dissociate. Fp then forms an insoluble aggregate, whereas Ip remains soluble. Reconstitution of active enzyme from the dissociated subunits has not yet been accomplished.

The amino acid composition of the SDH subunits of the two enzymes is very similar, although the mammalian enzyme is slightly larger. The bacterial Ip is less polar than the mammalian Ip (22). Structural and functional similarities between the two enzymes are also expressed in reconstitution experiments of the mammalian succinate-Q reductase and succinoxidase. The bacterial SDH can substitute for the mammalian enzyme to form a hybrid reductase (35) and oxidase (34), respectively.

Both Fp and Ip contain nonheme iron and acid-labile sulfur that, together with cysteinyl residues, are the building blocks of several iron-sulfur centers. These centers render soluble SDH sensitive to inactivation by oxygen. The enzyme should, therefore, be kept under anaerobic or reducing conditions in the presence of succinate. The exact stoichiometry, localization, and function of each iron-sulfur center in the mammalian enzyme are not known (4, 6, 14, 71, 84). Reports on the *Rhodopseudomonas sphaeroides* (47) and *R. rubrum* (11, 34) SDH suggest that photosynthetic bacteria have similar sets of iron-sulfur centers. The general view is that the Fp subunit contains two $Fe_2S^X_2$ (S^X indicates acid-labile sulfur) clusters, designated S-1 and S-2. Center S-1 is reduced by succinate. Center S-2 has a very low redox potential, and it can be reduced by dithionite. The Ip subunit probably contains a $Fe_2S^X_4$ HiPiP-type iron-sulfur center, designated S-3. Center S-3 is very susceptible to destruction by oxygen in the soluble enzyme. This center is essential for expression of the low K_m site for ferricyanide, and it is involved in the electron transport from succinate to quinone in the succinate-Q reductase. The substrate binding site of SDH is located in the Fp subunit (52). Reducing equivalents from the oxidation of succinate are transferred via the FAD to iron-sulfur center S-1, S-3, and ultimately to quinone. Electron transport to quinone can be inhibited by 2-thenoyltrifluoroacetone (35, 64, 87) or carboxanilides (e.g., carboxin) (64). Both inhibitors block electron transfer between center S-3 and quinone, but they do not affect the reduction of center S-3 by succinate. Purified SDH has no Q reductive activity, and SDH activity is not inhibited by 2-thenoyltrifluoroacetone or carboxin. The binding site for these electron transfer inhibitors has been suggested to involve both SDH and a membrane component (15, 35). Recent results by Ramsay et al. (78) indicate that SDH does not bind carboxin. Purified beef heart succinate-Q reductase was photoaffinity labeled with a carboxin analog carrying an azido group. The azidocarboxin was preferentially linked to the hydrophobic polypeptides C_{II-3} + C_{II-4} but not to the SDH subunits. Isolated C_{II-3} and C_{II-4} were not labeled by azidocarboxin.

GENETICS

Well-characterized mutants are powerful tools in studies on the arrangement and control of structural genes and also in the elucidation of enzyme structures and mechanisms of enzyme action. Different methods have been used for the isolation of SDH mutants in bacteria. *E. coli* SDH mutants can be enriched and selected for by their ability to grow on fumarate, but not on succinate, as the sole carbon source (45). In *Agrobacterium tumefaciens*, an SDH negative mutant was found among mutants able to grow on hexoses but not on Krebs cycle intermediates or pyruvate (13). A specific and elegant method to obtain *E. coli* SDH mutants is to use α-ketoglutarate dehydrogenase mutants. These mutants cannot grow aerobically in a glucose minimal medium without succinate or lysine and methionine. However, double mutants that lack both SDH and α-ketoglutarate dehydrogenase activity can grow on glucose alone (19). The reason is that the low intracellular concentration of succinate that is needed for biosynthetic purposes is depleted by SDH in α-ketoglutarate dehydrogenase mutants. When SDH is inactivated by mutation, the succinate level in the α-ketoglutarate dehydrogenase mutant will be high enough to permit growth on glucose alone. *E. coli* SDH mutants show a low SDH activity due to the membrane-bound fumarate reductase working in reverse (89).

For isolation of *B. subtilis* SDH mutants acid accumulation (12) and defective sporulation (27, 83) of mutants defective in Krebs cycle enzymes have been exploited. Sporulation defects are associated with characteristic pigmentation and colony morphology. Acid-producing bacterial colonies can be identified on plates containing calcium carbonate by the formation of halos around the colony or by a change in color on plates containing a pH indicator. After primary selection of acid-producing bacteria, SDH mutants are identified by the in vivo accumulation of radioactive succinate when grown in the presence of radioactive glutamate (27, 83), or by in

vitro SDH zymogram staining of lysed bacterial colonies by a replica technique (25). The SDH negative phenotype is finally confirmed by in vitro SDH assay of membrane preparations. The kinds of mutants obtained are of course influenced by the selection procedure and the method used for identifying the defect. For instance, a mutant may have a defective SDH in vivo but have a normal SDH activity with artificial electron acceptors in vitro.

E. coli mutants capable of growth on fumarate but not on succinate and lacking in vitro SDH activity were isolated by Spencer and Guest (90). Of 84 mutants, 74 had mutations in the *sdh* locus at 16.2 min on the *E. coli* chromosomal map (18). Four of the *sdh* mutations were nonsense, as they could be suppressed by a glutamine inserting amber suppressor gene. The polypeptide composition of cytoplasmic membranes from two nonsense mutants was analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A polypeptide, M_r 67K, was missing in the mutants, but was present in the suppressed mutants and the wild type. This polypeptide is most probably the Fp subunit of SDH. The experimental results strongly suggest that the *sdh* locus in *E. coli* contains the structural gene for the Fp polypeptide.

All SDH-negative mutants isolated in *B. subtilis* carry mutations in the *citF* locus located at 255° on the chromosomal genetic map (44, 69, 83). Two mutants with reduced in vitro SDH activity have been described, and they have correspondingly reduced amounts of SDH protein. The respective mutations do not map in the *citF* locus (69, 82). The relative order of 11 *citF* mutations has been established by transformation crosses (69; manuscript in preparation). The presence and location of Fp and Ip have been determined in each mutant by using subunit-specific antibody (41), and the cytochrome spectra of the mutant membranes have been determined. One mutant, *citF101*, contains an inactive, membrane-bound SDH complex, i.e., Fp, Ip, and cytochrome b_{558} (see below, Membrane Binding). The remaining 10 mutants either totally lack the Fp or Ip subunits (or both) or have one or both of them located in the cytoplasm. No SDH enzyme activity was found in the cytoplasmic fraction of any *citF* mutant. Based on the phenotypes of the *citF* mutants and the position of the respective mutations, the *citF* locus has been divided into three functional regions (Fig. 1). Mutations in the left region (*citF78* to *citF12*) all contain cytoplasmic Fp and Ip. The three leftmost mutants lack spectrally detectable cytochrome b_{558}. These mutants are unable to bind Fp and Ip to membranes. This region of the *citF* locus is thought to contain the structural gene for apocytochrome b_{558}. Mutations which lie between the left and middle region may affect either cytochrome b_{558} or the Fp subunit. The middle region of the *citF* locus (*citF69* to *citF2*) encompasses mutants which contain cytochrome b_{558} but which lack Fp and probably Ip. This region may code for the Fp subunit. Finally, the right region (*citF103* and *citF83*) is characterized by mutants which contain cytochrome b_{558} and cytoplasmic Fp but lack Ip. Consequently, this region is suggested to contain the structural gene for the Ip subunit.

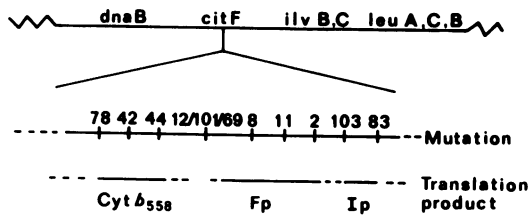

FIG. 1. *Present genetic map of the citF locus in B. subtilis and the proposed citF gene products.*

In summary, it is suggested that the *citF* locus contains the structural genes for each of the three subunits of the SDH complex, and that their order from left to right is cyt*b*-Fp-Ip (Fig. 1). Little is known about the genetic control of SDH synthesis in *B. subtilis* or in any other organism. Recently, we have studied two mutants which lack all three subunits of the SDH complex. One of these mutants (isolated and kindly provided by S. A. Zahler) has phage SP-beta integrated into the *citF* locus. The other mutant, obtained from ethylmethanesulfonate-treated spores by the method described by Ito and Spizizen (48), most likely carries a point mutation in the *citF* locus since it can revert to wild type. The properties of the above mutants support the notion that *citF* contains the structural genes for each of the three subunits of the SDH complex and that these genes are coordinately controlled.

Saccharomyces cerevisiae mutants unable to grow on nonfermentable substrates and which have a very low in vitro SDH activity were isolated by De Kok et al. (23). Two mutants had low levels of covalently bound FAD and iron-sulfur center S-3 in the mitochondrial inner membrane. These mutants carried allelic nuclear mutations and most probably lack membrane-bound Fp and Ip subunits. Another mutant, belonging to another complementation group, had a less severe reduction of covalently bound FAD. This mutant may contain a membrane-bound, inactive SDH.

Another approach to SDH genetics is to isolate mutants resistant to specific enzyme or electron transport inhibitors. The fungicide carboxin has been shown to inhibit mitochondrial (64, 95) as well as bacterial (94) electron transport from SDH to quinone. Mutants with in vitro carboxin-resistant SDH activity were isolated from the obligate aerobic ascomycete *Aspergillus nidulans* and the basidiomycete *Ustilago maydis*. Mutations in at least three different nuclear genes conferred resistance to carboxin in *A. nidulans* (31). It is unclear how SDH activity was actually measured, but as carboxin inhibited the SDH activity almost completely in the wild-type membranes, SDH was probably measured with ferricyanide as the electron acceptor. A similar chromosomal mutation in *U. maydis* resulted in a carboxin-resistant, succinate-dependent reduction of ferricyanide or DCIP (28, 29). The mutation has probably decreased the affinity of the binding site for the inhibitor. However, as it is not known whether this binding site is wholly or partly situated on SDH or on some other membrane component, it cannot yet be concluded that the above mutations are located in the SDH structural genes.

MEMBRANE BINDING

Integral membrane proteins are hydrophobic or have hydrophobic domains which penetrate the lipid bilayer of the membrane. Such proteins can only be extracted by procedures which destroy the integrity of the membrane. Detergents are commonly used to break up membranes, and the core lipid surrounding the hydrophobic part of a membrane protein can be substituted for by a detergent micelle. The solubilized protein thus binds detergent, and it will aggregate on removal of detergent. Hydrophilic, water-soluble proteins generally do not bind detergent. Detergent binding and solubility can thus be used to operationally classify proteins as hydrophobic or hydrophilic (92).

SDHs isolated from *R. rubrum* chromatophore membranes and beef heart mitochondrial inner membranes are both soluble proteins with polarity indexes above 40% (22). The mitochondrial enzyme shows some dimerization at high protein concentrations (16), but the sedimentation constant of the enzyme in the ultracentrifuge is not changed by the presence of the nonionic detergent Triton X-100 or the more powerfully disaggregating ionic detergents sodium dodecyl sulfate and cetyldimethylethylammonium bromide indicating no detergent binding and no aggregation of SDH. Based on the criteria of detergent binding, polarity index, and solubility, purified SDH does not have the characteristics of an integral membrane protein. Reconstitution experiments (8, 32) and experiments with cytochrome deficient mutants (41, 46), discussed later in this article, strongly suggest that SDH is bound to specific limiting sites in the membrane, rather than by hydrophobic interaction with the lipid bilayer. The specific component(s) involved in anchoring SDH to the membrane should be hydrophobic and require detergent for solubilization. Proteins interacting mainly nonhydrophobically with SDH in the membrane should be solubilized by nonionic detergent with SDH still attached to them. Ideally, there will be one protein per micelle when solubilization is done in the presence of excess detergent micelles (92).

Triton X-100 treatment of *Neurospora crassa* mitochondrial inner membranes at low ionic strength results in the solubilization of a monodisperse succinate-Q reductase. Each reductase molecule binds one detergent micelle and is composed of three different subunits (99) (Table 3). In addition to Fp and Ip subunits of SDH, the reductase contains a low-molecular-weight (M_r, 14K) cytochrome *b*. The cytochrome is the detergent binding, hydrophobic part of the reductase (H. Weiss, personal communication). A similar monodisperse SDH-cytochrome *b* complex containing SDH and a detergent binding (unpublished experiments) cytochrome b_{558} polypeptide in an equimolar amount to SDH has also been isolated from Triton X-100-solubilized *B. subtilis* membranes (39, 40) (Table 3). It is not known if this complex has quinone reductase activity.

Mammalian succinate-Q reductase (complex II) extracted with bile acids from beef heart mitochondria contains four polypeptides. Two of these are the SDH Fp and Ip subunits. Complex II also contains a low molecular weight cytochrome b_{560} which possibly is the C_{II-3} polypeptide. The fourth polypeptide is called C_{II-4} (9, 36, 38). The four polypeptides are present in equimolar amounts. Also, the molar ratio of covalently bound FAD to protoheme is about 1 in complex II (38). The apparent molecular weight obtained in sodium dodecyl sulfate-polyacrylamide gel electrophoresis of C_{II-3} and C_{II-4} is strongly influenced by the buffer system used in electrophoresis (9). These polypeptides have a high content of apolar amino acids, indicating that they are quite hydrophobic (35). Most probably, the smallest polypeptides found in the detergent-solubilized SDH complexes from *N. crassa*, *B. subtilis*, and beef heart mitochondria are integral membrane proteins.

The labeling of mitochondria and submitochondrial particles, which are inside-out mem-

TABLE 3. *Characterized SDH-cytochrome b complexes*[a]

Organism or organelle	Detergent used to solubilize complex	Quinone reductase activity	Mol wt (K) of:				α-Absorption maximum of cytochrome (nm at 25°C)
			Fp	Ip	Cytochrome *b*		
					C_{II-3}	C_{II-4}	
B. subtilis[a]	Triton X-100	Not determined	65	28	19	Absent	558
N. crassa[b]	Triton X-100	Present	72	28	14	Absent	559
Beef heart mitochondria[c]	Bile salt	Present	70	27	17 to 13.5	14 to 7	560

[a] *B. subtilis* data from references 39 and 40.
[b] *N. crassa* data from reference 99.
[c] Beef heart mitochondria data from references 9 and 38.

brane vesicles, with the water-soluble, membrane impermeable reagent ^{35}S-labeled diazobenzene sulfonate results in incorporation of label into C_{II-3} in mitochondria (63). Photoaffinity labeling of complex II in egg lecithin vesicles with (arylazido) phospholipids results in cross-links between reagent and polypeptides C_{II-3} and C_{II-4} (30). Also, the Ip, but not the Fp subunit of SDH, is linked to the hydrophobic reagent. Cross-links to these complex II polypeptides with the (arylazido) phospholipids occur to approximately the same relative extent whether the reactive nitrene group is located in the head group region or at the CH_3 end of one of the fatty acid chains. Peptide C_{II-4} can be bound to SDH in a stoichiometric amount also in the absence of C_{II-3} (1). However, the C_{II-3} component is necessary for expression of beef heart succinate-Q reductase activity. Together, these findings on complex II indicate that the C_{II-3} and C_{II-4} polypeptides penetrate into the membrane phospholipid bilayer. The C_{II-3} polypeptide is exposed on the outer surface of the mitochondrial inner membrane and spans the membrane to functionally interact with SDH (probably the Ip subunit) either directly or via C_{II-4} on the inside (30).

RECONSTITUTION OF MEMBRANE-BOUND SDH

The structural and functional combination of purified soluble SDH with soluble or particulate components of the respiratory chain is called reconstitution. The first successful reconstitution was reported by Keilin and King (51), who reconstituted succinoxidase activity from soluble SDH and an alkali-treated heart muscle preparation. Alkali treatment (pH 9.3 at 38°C under argon) of beef heart mitochondrial ETP or complex II inactivates both succinate-PMS and succinate-Q reductase activity. SDH is protected by the presence of succinate in the incubation buffer. The loss of activity after alkali treatment is not due to release of SDH since most of the SDH-flavin remains bound to the particles. SDH is released from ETP treated at pH 10.0, but the reconstitutive activity of the particles is destroyed at this pH. When reconstitutively active SDH is added to alkali-treated ETP or complex II, the enzyme is bound to the particulate preparations, which then regain succinoxidase and succinate-Q reductase activity, respectively. Particles reconstituted with excess SDH contain twice as much SDH-flavin as originally present (32, 88). In *R. sphaeroides* chromatophore membranes, SDH activity and iron-sulfur center are removed by a single wash at pH 9.1 under anaerobic conditions and with succinate in the buffer (47). This treatment does not destroy the integrity of the chromatophore membranes, but succinoxidase activity is abolished. When the released SDH is added back to the alkali-treated membranes, succinoxidase activity is reconstituted. Also, in *Mycobacterium phlei*, SDH can be dissociated from ETP by alkali treatment under argon with subsequent loss of succinoxidase activity (49). The released SDH was fractionated by $AmSO_4$ and chromatography on hydroxyapatite. Neither the purity nor the composition of the released *M. phlei* SDH was reported. Succinoxidase activity could be reconstituted by addition of the SDH preparation to *M. phlei* ETP treated with alkali or silicotungstate.

The chaotropic ion perchlorate has been used successfully to selectively release SDH in a reversible manner from beef heart complex II and from *R. rubrum* chromatophore membranes, respectively (22, 37). A reconstitutively active SDH is obtained when extraction is made in a reducing environment and in the presence of succinate. The perchlorate-extracted *R. rubrum* SDH can reconstitute a hybrid succinoxidase when mixed with alkali-treated beef heart submitochondrial particles (34). Also, the mammalian enzyme can interact with alkali-treated *R. rubrum* chromatophore membranes, but in this case reconstitution is less efficient than with the

homologous membrane. Attempts to extract SDH from *B. subtilis* (unpublished data) or *Micrococcus lysodeikticus* (17) membranes with perchlorate have been unsuccessful. Generally, purified SDH is found to give better reconstitution when it is prepared fresh, protected against oxygen, and kept in the presence of succinate. The reconstitutive activity of SDH does not correlate with the succinate-PMS activity of the soluble enzyme. When purified SDH is exposed to oxygen the reconstitutive activity decays faster than does the succinate-PMS activity. SDH solubilized and purified with different methods often has similar activities in the PMS assay, but greatly different reconstitutive activities as has been observed both with mammalian and bacterial SDH (3, 49, 88). An explanation for this can be found in the observation that in the mammalian enzyme, the S-3 iron-sulfur center, the low-K_m ferricyanide activity and the reconstitutive activity decay in parallel under aerobic conditions (96). This suggests that the integrity of S-3 is necessary for reconstitution. This iron-sulfur center is very fragile in the soluble enzyme, but it is stable in the particulate enzyme where it is protected against oxygen inactivation. The fact that SDH has a low-K_m ferricyanide site exposed in the soluble enzyme and hidden in the particulate enzyme can be used to differentiate between the two states of the enzyme. Different preparations from beef heart mitochondria that can bind SDH and protect center S-3 against oxygen inactivation and that are active in reconstitution of 2-thenoyltrifluoroacetone-sensitive succinate-Q reductase have been described by several groups (1, 35, 97, 101, 102). All of these preparations are particulate and contain one or both of the low-molecular-weight complex II polypeptides C_{II-3} and C_{II-4}, various amounts of cytochrome *b*, and phospholipids. Hatefi and Galante (35) isolated a cytochrome *b* preparation which contains both C_{II-3} and C_{II-4} and a stoichiometric amount of cytochrome b_{560}. The protoheme binding component was not identified. Reconstitution of succinate-Q reductase with this preparation involves a structural association between cytochrome *b* and SDH. The cytochrome b_{560} interacts electronically with both SDH and quinone. These results indicate both a structural and a functional role of cytochrome b_{560} in succinate-Q reductase. Ackrell et al. (1) described a preparation similar to that isolated by Hatefi and Galante, but with a lower cytochrome *b* content. Chymotrypsin treatment selectively removes the C_{II-3} polypeptide. It was further shown that C_{II-4} binds SDH in the absence of C_{II-3}, but the latter polypeptide is essential for succinate-Q reductase activity. A polypeptide called QPs which shows succinate-Q reductase activity when mixed with soluble, reconstitutively active SDH was described by Yu and Yu (101, 102). The most highly purified QPs gives only one main band, M_r 15K, in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and has a low activity in reconstitution. Another QPs preparation with high reconstitutive activity contained two main polypeptides (M_r 17K and 15K, respectively) and some cytochrome *b*. Vinogradov et al. (97) have described a reconstitutively active preparation in which about 80% of the protein had a relative molecular weight less than 13K.

BIOSYNTHESIS AND MEMBRANE BINDING OF SDH IN *BACILLUS SUBTILIS*

Recently, information about biosynthesis and membrane binding in vivo of SDH has been obtained from studies of SDH and heme mutants of *B. subtilis* (41, 46). A 5-aminolevulinic acid (5-ala) auxotroph cannot make heme and, consequently, not cytochromes when grown without 5-ala. The strict aerobe *B. subtilis* apparently contains an excess of cytochromes because the 5-ala auxotroph grows at an undiminished rate for about three generations without 5-ala. Membranes isolated from 5-ala-starved auxotrophs have a strongly reduced level of cytochromes, especially cytochromes *b* and *c* (46). Bulk membrane protein synthesis proceeds at an undiminished rate during the first three generations of growth of a 5-ala auxotroph in 5-ala-free medium (46). However, no membrane-bound SDH is made, and there is no net increase in membrane-bound SDH activity. Fp and Ip subunits are still synthesized in the cytoplasm, but they are not associated and lack detectable enzymatic activity (41). When heme synthesis is allowed to resume, the cytoplasmic SDH subunits bind to the membrane at an initial rate several times higher than the growth rate of the bacteria (46), with a concomitant rise in membrane-bound SDH activity. Membrane binding of the soluble SDH subunits occurs also when protein synthesis is blocked by chloramphenicol. These results strongly suggest the presence of a limiting number of specific SDH-binding sites in the *B. subtilis* cytoplasmic membrane. Evidence that cytochrome b_{558} is (part of) this binding site is provided by the observations that (i) cytochrome b_{558} is present in stoichiometric amounts in the Triton X-100-solubilized, purified membrane SDH complex (39, 40) and (ii) mutants which specifically lack cytochrome b_{558} contain the SDH Fp and Ip subunits in the cytoplasm

(41). A model for biosynthesis and membrane binding of SDH in *B. subtilis* is shown in Fig. 2. Fp and Ip subunits are synthesized as soluble polypeptides and are not associated in the cytoplasm. The soluble subunits are precursors to the membrane-bound enzyme (41). The soluble subunits have the same mobility in sodium dodecyl sulfate-gel electrophoresis as the membrane-bound SDH subunits (40, 41). It is probable that apocytochrome b_{558} is inserted into the membrane, e.g., during 5-ala starvation. In reconstitution experiments with hemeless mutants it has been shown for *Staphylococcus aureus* that the apocytochrome of *b*-type cytochromes is inserted into the membrane probably in connection with apocytochrome synthesis (59). Insertion of protoheme into membrane-bound apocytochrome *b* in *B. subtilis* is suggested to expose a binding site for the Fp and Ip subunits of SDH. FAD is covalently added to the Fp subunit before membrane binding (41). It is not known when the iron-sulfur centers are incorporated into the subunits; consequently, their role in membrane binding of SDH is uncertain. The isolation of *B. subtilis* mutants with enzymatically inactive, membrane-bound SDH rules out the possibility that enzyme activity is essential for membrane-binding (unpublished experiments). The tight association of the Fp and Ip subunits in soluble SDH isolated from beef heart mitochondria or *R. rubrum* is in apparent contrast to the free precursor subunits found in the *B. subtilis* cytoplasm. Also, the insolubility of Fp after chaotrop-induced dissociation of the two subunits of the soluble mammalian or *R. rubrum* SDH contrasts with the soluble Fp subunit found in *B. subtilis*. This may indicate that the interaction between the two subunits is different in different species. Since the cytoplasmic subunits are precursors to the membrane-bound enzyme, it is also possible that they lack iron-sulfur centers and that these are required for the subunits to assume a conformation which promotes their tight association. In vitro reconstitutions of isolated Fp and Ip subunits has not been accomplished with SDH from any organism. Interestingly, Hanstein et al. (32) have speculated that the Fp subunit alone could express fumarate reductase activity and that the Ip subunit functions to shift the equilibrium in a direction favoring succinate oxidation.

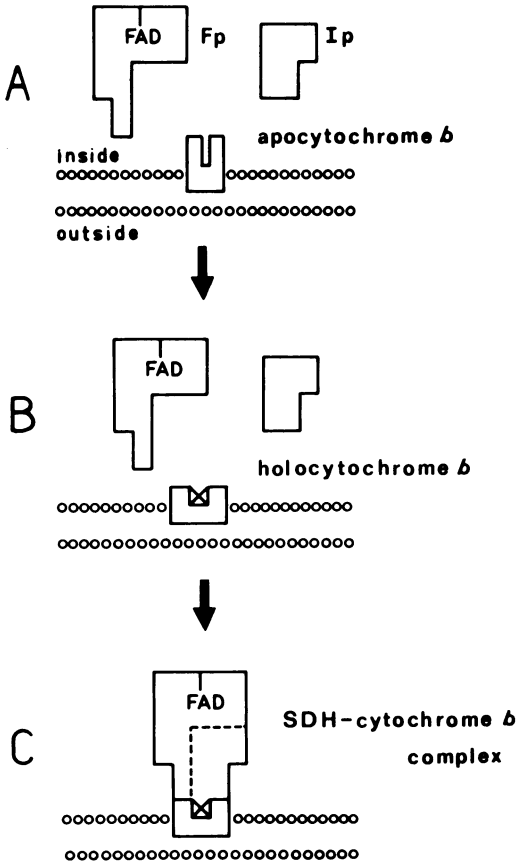

FIG. 2. *Model for the synthesis and assembly of the B. subtilis SDH-cytochrome b_{558} complex. The Fp and Ip subunits are synthesized as soluble proteins. FAD is covalently bound to the soluble Fp subunit. Apocytochrome b is inserted into the membrane in connection with its synthesis (A). As protoheme is bound to apocytochrome b a binding site(s) for the Fp and Ip subunits is exposed (B), which is rapidly followed by the assembly of a functional membrane-bound SDH-cytochrome b_{558} complex (C).*

SUMMARY AND SOME PERSPECTIVES

The structure of membrane-bound SDH seems to be quite similar in such widely different species as cow, bakers' yeast (67), *N. crassa*, *R. rubrum*, and *B. subtilis*. In all of these species the enzyme consists of two unequal subunits, a larger flavoprotein and a smaller polypeptide which, at least in beef heart and *R. rubrum*, is an iron-sulfur protein. Most likely SDH has a similar structure also in *E. coli* (90, J. L. Cowell, M. Raffeld, and I. Friedberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, 157, p. 100) and *M. lysodeikticus* (P. Owen, personal communication). The very similar amino acid composition of SDH from beef heart mitochondria and from *R. rubrum* chromatophore membranes suggests considerable evolutionary conservation in the enzyme. On the other hand, interaction of SDH with a particular membrane and the nature of the components that serve to bind the enzyme

to that membrane seem quite different in different species.

There have been numerous attempts to purify SDH from various bacterial species (42, 43, 53, 58, 60, 61, 76, 77, 79). Common to most of this work is the great difficulty experienced in trying to separate SDH from cytochrome. Before the structure of beef heart SDH was elucidated, it was actually proposed that in *Corynebacterium diphtheriae* SDH was a *b* cytochrome (75). In some partially purified, cytochrome *b*-containing, bacterial SDH preparations, the cytochrome is reduced by succinate (39, 42, 58, 61, 76), whereas in others it is not (77). Purified membrane-bound SDH from *N. crassa* is structurally very similar to the purified *B. subtilis* SDH complex. However, in the *N. crassa* complex the cytochrome *b* has not been shown to be reduced by succinate. In beef heart mitochondria two polypeptides seem required to give a fully functional membrane-bound SDH (see above, Membrane Binding). The C_{II-3} polypeptide, or an M_r 9K fragment thereof, is necessary for the expression of succinate-Q reductase activity, whereas polypeptide C_{II-4} alone seems sufficient for membrane binding of SDH (1). To our knowledge there are only two reports of binding of SDH in vivo to a membrane which lacks cytochrome *b*. Chromatophore membranes from *Chromatium* sp. strain D grown heterotrophically with succinate as carbon source have a high SDH activity, although the membranes do not contain protoheme, the characteristic prosthetic group of all *b* type cytochromes (20). A yeast mutant that required 5-ala to make cytochrome contained about 25% of the enzyme activity when starved for 5-ala compared with cells grown with 5-ala (100).

SDH is located on the cytoplasmic side of the cytoplasmic membrane in bacteria and on the matrix side of mitochondrial inner membranes. There is an important difference, however, between the biogenesis of SDH in eucaryotic and procaryotic cells. In eucaryotic cells, or at least in mammals (62), bakers' yeast (23), and *N. crassa* (25), the SDH gene(s) is nuclear. Since the mitochondrial protein-synthesizing machinery is completely separated from that of the rest of the cell, SDH has to be synthesized in the cytoplasm and then transported through the outer and inner mitochondrial membrane, to be attached ultimately to the matrix side of the inner membrane. In bacteria there is no such transport problem, and both subunits are synthesized, assembled, and membrane-bound on the same side. Soluble cytoplasmic and enzymatically active SDH, which is suggested to be a precursor of mitochondrial SDH, has been found in pea cotelydons (65) and in yeast (5). The function of SDH as a Krebs cycle enzyme is similar in both procaryotic and eucaryotic cells. However, the evolution of cell organelles and membrane systems in eucaryotic cells may have favored alternative and more complex solutions to the problems of membrane binding of SDH and transfer of electrons from succinate oxidation to the respiratory chain than the seemingly simplest one of direct structural and functional coupling between SDH and cytochrome.

We think that future work on SDH will focus to a large extent on the structure, membrane topology, and biogenesis of the enzyme as well as the succinate-Q reductase complex. Among the problems to be solved are the following. (i) How are the SDH structural genes organized and controlled? (ii) How and when are the prosthetic groups (flavin and the iron-sulfur centers) incorporated in SDH? (iii) What are the factors involved in membrane binding of SDH? Some of these and other problems can only be resolved by using organisms in which there exist well-developed genetic systems.

ACKNOWLEDGMENTS

We are grateful for the patient secretarial help provided by Ruth Du Rietz, Birgit Sehlberg, and Yvonne Åström.

Work done in our laboratory was supported by grants from the Swedish Medical Research Council and from Karolinska Institutet.

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